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Pyridostigmine Synergistic Toxicity Study

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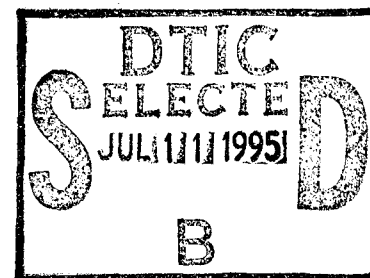
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EXECUTIVE SUMMARY
TOXICOLOGICAL STUDY 75-48-2665
ACUTE ORAL TOXICITY STUDY OF PYRIDOSTIGMINE BROMIDE,
PERMETHRIN, AND DEET IN THE LABORATORY RAT

1. **PURPOSE:** The purpose of this study was to determine potential toxic interactions when pyridostigmine bromide, permethrin, and DEET are given concurrently to male rats by gavage.

2. **FINDINGS:** There is a significant increase on the lethal effect in rats given pyridostigmine bromide, permethrin, and DEET simultaneously by gavage when compared to expected additive lethal effect of the individual compounds.

3. **MILITARY STATUS:**

a. *Pyridostigmine bromide:* Pyridostigmine bromide (PB) is a quaternary ammonium carbamate which has been approved by the Food and Drug Administration (FDA) as a treatment for myasthenia gravis (Mestinon) and for the reversal of nondepolarizing neuromuscular blocking agents (Regonol). A new drug application (NDA) has been submitted by the DOD to the FDA for the indication of pretreatment for nerve agents. PB was used in the Gulf War at a dose of one 30mg tablet every eight hours (90mg per day). Pyridostigmine bromide was taken for about two weeks at the start of the air war in mid January and again at the start of the ground war in mid February. The majority of service members in the theater of operations took some PB during these periods.

b. *Permethrin:* Permethrin, a third generation synthetic pyrethroid, has been approved for use as an insecticide by the Environmental Protection Agency (EPA). This compound has also been used to impregnate army battle-dress uniforms in the field. Formulations for application include a 0.5% aerosol and a 40% solution applied with either a 2-gallon compressed air sprayer or via a passive absorption method in a plastic bag (for uniform impregnation). Although a factory impregnation method is planned, this method has not yet been used to impregnate any battle dress uniforms. The only impregnation method available to soldiers prior to and during the Gulf War was the aerosol spray can method. The aerosol spray cans received only limited distribution within the theater (less than 5% of deployed units had distributional access). The aerosol spray can method of impregnation provides protection for only about six washings, or about six weeks of use.

c. *DEET:* DEET is an EPA approved insect repellent which is widely used commercially. Formulations prepared for the U.S. Army include a 75% DEET in ethanol, 33% extended duration formulation, and 19% in stick. Entomologists assigned in the Gulf during the conflict indicated a very low usage of personal repellents, including DEET, even at times and in areas where mosquitos were present and biting. Also, the cool seasonal climatic conditions which prevailed at the time of the war (January and February, 1991) resulted in the near absence of biting insects. In fact,

insect biting rates dropped to insignificant or near zero levels in October and did not rise again until the following March.

4. ABSTRACT: This study investigated the lethal interaction of pyridostigmine bromide (PB), permethrin, and DEET when given to adult male rats by gavage. The study was separated into two phases. The first (Phase I) determined the acute oral lethal dose-response relationship of each compound with the vehicle, propylene glycol. The second (Phase II) was divided into two portions. One portion (positive control) was a dose-response study using probit units obtained from phase I (LD 16, 30, 50, 70, and 84). Dosage solutions for the second (interaction) portion of phase II contained the calculated LD₁₆ (additive LD₃₂) of two compounds while the concentration of the third compound was varied. Rats were fasted overnight, dosed, and observed for 14 days. A significant increase in lethality occurred when PB, permethrin, and DEET were given concurrently when compared to additive values. Furthermore, solutions containing PB and permethrin or PB and DEET also caused a significant increase in lethality when compared to expected additive values. This information suggests that lethality in this study was more than an additive effect. Dosage levels of compounds used in this study were sufficient to produce lethality following a single dose, and were far in excess of conceivable human exposure levels. For example, in order for an average 70kg (155lb) service member to become exposed to the lowest doses used in this study (PB = 46mg/kg, Permethrin = 279mg/kg, DEET = 1946mg/kg), this person would have to simultaneously ingest 107 PB tablets (30mg each), 23 six ounce cans of 0.5% permethrin aerosol spray, and 6.6 two ounce tubes of 33% DEET. Human exposure would most likely occur at low levels over an extended period of time and by differing routes.

Table I: Calculations of the equivalent exposure values for an average 70kg service member for compounds used in this study based on the following information: Pyridostigmine bromide - 30mg tablet; Permethrin - six ounce cans of 0.5% aerosol spray; DEET - two ounce tubes of 33% DEET.

	LD ₁₆	LD ₃₀	LD ₅₀	LD ₇₀	LD ₈₄
Pyridostigmine	107 tablets	122 tablets	143 tablets	177 tablets	194 tablets
Permethrin	23 cans	42 cans	83 cans	161 cans	296 cans
DEET	6 tubes	9 tubes	12 tubes	17 tubes	23 tubes

TOXICOLOGICAL STUDY 75-48-2665
ACUTE ORAL TOXICITY STUDY OF PYRIDOSTIGMINE BROMIDE,
PERMETHRIN, AND DEET IN THE LABORATORY RAT

1. REFERENCES: See Appendix A for a list of references.
2. AUTHORITY: Memorandum of agreement. (August 19, 1994)
3. BACKGROUND:

a. *General*: The objective of this study was to determine potential lethal toxic interactions when pyridostigmine bromide, permethrin, and DEET are given concurrently to male rats by gavage. This study was requested by U.S. Army Medical Research and Materiel Command.

b. *Pyridostigmine bromide*: Pyridostigmine bromide (PB) is a quaternary ammonium carbamate which has been approved by the Food and Drug Administration as a treatment for myasthenia gravis (Mestinon) and for the reversal of nondepolarizing neuromuscular blocking agents (Regonol). A new drug application (NDA) has been submitted by the DOD to the FDA for the indication of pretreatment for nerve agents. Its use in the Gulf War consisted of a dose of one 30mg tablet every eight hours (90mg per day). PB, and other carbamates, are reversible inhibitors of both acetylcholinesterase (true cholinesterase) and nonspecific esterase (pseudocholin-esterase). Quaternary carbamates do not easily pass the blood brain barrier and their effect, therefore, is primarily on the peripheral nervous system (Taylor, 1991). Metabolic and kinetic studies indicate that PB is poorly absorbed from the gut and, in order to produce a pharmacological effect, high levels must be given orally (Cohan *et al*, 1976; Somani *et al*, 1972). The reported acute oral median lethal dose for PB in the rat is 80 mg/kg (Hane, 1977).

c. *Permethrin*: Permethrin, a third generation synthetic pyrethroid, has been approved for use as an insecticide by the Environmental Protection Agency (EPA). It is the constituent of many household and agricultural insecticidal formulations. This compound has also been used to impregnate army battle-dress uniforms in the field. Formulations for application include a 0.5% aerosol and a 40% solution applied with either a 2-gallon compressed air sprayer or via a passive absorption method in a plastic bag (for uniform impregnation). Although a factory impregnation method is planned, this method has not yet been used to impregnate any battle dress uniforms. The only impregnation method available to soldiers prior to and during the Gulf War was the aerosol spray can method. The aerosol spray cans received only limited distribution within the theater (less than 5% of deployed units had distributional access). The aerosol spray can method of impregnation provides protection for only about six washings, or about six weeks of use. Permethrin and other type I pyrethroids produce their toxic effect primarily by their action on the Na^+ channel. Other toxic mechanisms associated with synthetic type I pyrethroids include inhibition of $\text{Na}^+/\text{Ca}^{++}$ ATPase, $\text{Ca}^{++}/\text{Mg}^{++}$ ATPase, and calmodulin (Clark and Matsumura, 1982). The reported acute oral median lethal dose for technical permethrin in the male rat is 3801 mg/kg, however, factors such as

gender, nutritional state, vehicle, and strain of rat may lower the LD₅₀ by an order of magnitude (Metker *et al.* 1977).

d. *DEET*: DEET is an EPA approved insect repellent which is widely used commercially. Several formulations using various concentrations of DEET are available. It is the active ingredient in products such as Deep Woods Off and Cutter Insect Repellent[®]. Formulations prepared for the U.S. Army include a 75% DEET in ethanol, 33% extended duration formulation, and 19% in stick. Entomologists assigned in the Gulf during the conflict indicated a very low usage of personal repellents, including DEET, even at times and in areas where mosquitos were present and biting. Also, the cool seasonal climatic conditions which prevailed at the time of the war (January and February, 1991) resulted in the near absence of biting insects. In fact, insect biting rates dropped to insignificant or near zero levels in October and did not rise again until the following March. Although the exact mechanism of DEET toxicity is unknown, pathological findings indicate that this compound is a demyelinating agent which causes spongiform myelinopathy, primarily of the cerebellar roof nuclei (Verschoyle *et al.* 1992). The reported acute oral median lethal dose for DEET is 3287 mg/kg in the male rat (Macko and Weeks, 1980).

e. *Safety testing*: Safety testing on PB, provides the framework for a New Drug Application (NDA) submitted to the Food and Drug Administration (FDA) by the U.S. Army. Exhaustive safety studies for this drug have been performed in connection with its FDA approval for use in the treatment of myasthenia gravis and for the reversal of effects from nondepolarizing muscle relaxants. These studies include acute, subacute, subchronic and chronic trials in animals and humans. Absorption, distribution, and elimination studies for PB have been performed in connection with human clinical applications (Somani *et al.* 1972; Cohan *et al.* 1976). A comprehensive summary of these studies is included in the NDA (pp 107-174). Initial safety testing on PB was performed by Pharmacology Research, Inc. (Latvin and Sloane, 1954) and by Roche Laboratories (Pool and Hane, 1972; Hane, 1977).

Safety testing on DEET and permethrin has been performed for the U.S. Army by the Toxicology Division, Army Environmental Hygiene Agency. A review of the literature on toxicity of DEET is available through the Environmental Protection Agency's (EPA) Pesticide Registration Standard (1980). A similar review for permethrin is available through the World Health Organization's (WHO) Environmental Health Criteria 94 (1990).

4. MATERIALS:

a. *Chemical Compounds*:

Pyridostigmine bromide is a white crystalline powder with a molecular weight of 261.14. Neat PB used in this study (PB: WYR#250710BD) was obtained from Walter Reed Army Institute of Research (Washington, DC). Compound analysis, which was performed by SRI International (Lim *et al.* 1990), indicated that the PB used in this study was 99.1% pure.

Technical grade permethrin (3-(phenoxyphenyl) methyl (+)-*cis, trans*-3-(2,2-dichloroethenyl)-2,2-dimethylchloro-propanecarboxylate) is an amber colored liquid

with a pungent odor. The molecular weight of technical grade permethrin is 391.29 and it has a specific gravity of 1.19 to 1.27 at 20°C. Technical grade permethrin for this project was obtained from Coulston Products (Easton, PA). The compound was 91.6% pure with a *cis* isomer percentage of 42.3.

Technical DEET (N,N-Diethyl-*m*-toluamide) is a clear amber liquid with little or no odor. The molecular weight of DEET is 191.26 and it has a specific gravity of 0.996 at 20°C. Technical grade DEET used in this study was obtained from Mortflex, Inc. (Greensboro, NC) and contained 98.5% of the *meta* isomer.

The vehicle used in this study was propylene glycol (PG; 1,2-propanediol; Sigma Chemical Co., St. Louis, MO).

b. *Animals*: Male Sprague-Dawley rats were obtained from Charles River, Inc. (Raleigh, NC). The animals were individually housed in wire bottom cages (17.5 cm x 17.5 cm x 22 cm) and given feed (Zeigler Small Animal Feed; Zeigler Bros., Inc., Gardners, PA) and water *ad libitum*. Animal rooms were maintained at a constant temperature (69-73°F) and humidity (40-60%) as well as a twelve hour light/dark cycle. Animals were quarantined for two weeks prior to dosing and weighed between 250 and 300 grams at time of use.

5. METHODS:

a. *Brief overview*: This study was separated into two phases. The first phase was the development of the acute oral lethal dose-response relationship of each compound with the vehicle, propylene glycol. Information obtained from the first phase was used to determine dosage levels for the second phase. Dosage levels for the second phase were set at probit units of 4, 4.5, 5, 5.5, and 6. These levels were chosen because they lie within one standard deviation of the mean. These points insure a greater precision than do points beyond one standard deviation (Finney, 1979).

The second phase was divided into two portions. One portion was a dose-response study using probit units obtained from phase I. This group served as a positive control for the interaction portion of the study and verified data obtained from the first phase of the study. The second (interaction) portion of phase II was similar to the control portion except that the vehicle contained the calculated LD₁₆ (additive LD₃₂) of the other two compounds.

The studies described were conducted under Good Laboratory Practice (GLP; 40 CFR 160 and 792 and amendments). The investigators and technicians adhered to *The Guide for Care and Use of Laboratory Animals* (U.S. Department of Health, Education, and Welfare Publication No. NIH 86-23, 1985).

b. *Compound preparation and analysis*: Compounds were formulated the day of administration. Neat material was weighed and diluted with propylene glycol to produce 50 ml of each dosage solution. Approximately 30 ml was used for animal treatment while the remainder was used for compound analysis.

Compound analysis was performed by the Military Unique and Special Chemistry Program (Directorate of Laboratory Science, USACHPPM). Samples of permethrin

and DEET were diluted in acetonitrile and analyzed using gas chromatography and a flame ionization detector. PB was analyzed using solid phase extraction and high performance liquid chromatography (Shih and Ellin, 1993).

c. *Study protocol:* For the phase I study, animals were sorted into 16 groups of approximately equal weight distributions with ten animals in each group. Dosage levels for each compound were based on the LD₅₀ value most often cited in the literature for each individual compound. This value was used as the midpoint of the dose range with two doses lower and two doses higher (PB and DEET: ± 0.1 and ± 0.2 log; permethrin: ± 0.2 and ± 0.4 log). One group of ten animals received the vehicle, propylene glycol, only. Animals were fasted overnight (approximately 18 hours) and compounds were administered the following morning. Solutions were administered by gavage at a volume of 10 ml/kg. Animals were observed hourly until the start of the dark cycle. Gross necropsy was performed on all animals as soon as possible after death. Animals surviving the fourteen day study period were euthanized with CO₂ and examined for gross pathological lesions.

For the second phase of the study, animals were sorted into 15 groups of approximately equal weight distributions with six animals in each group for the positive control portion. The interaction portion contained 18 groups of 10 animals each. Procedures used for phase II were similar to those of phase I. Animals in the positive control group were given increasing doses of individual compounds in propylene glycol by gavage using dosage levels obtained from phase I data. Animals in the interaction portion of phase II were also given increasing doses of individual compounds, however, the vehicle used in this portion of the study contained LD₁₆ (additive LD₃₂) of the remaining two compounds. Hourly examination of animals was performed until the daylight cycle ended. Animals were examined for gross pathological lesions as soon as possible after death. At the end of the 14 day study period, surviving animals were euthanized with CO₂ and gross necropsy was performed.

d. *Statistical analysis:* The data obtained from phase I were subjected to probit analysis (EPA probit analysis program, version 4.1). In phase II, distributions of lethality in treated animals were compared to the expected additive lethality caused by compounds in dosage solutions. Critical values for Z were obtained for probabilities less than 5% and 1% (Daniel, 1991). In addition, a chi-square analysis was performed on groups dosed with solutions containing only two compounds. Differences were considered significant if the probability of their occurrence was less than 5%.

6. RESULTS

a. *Phase I:* The median lethal dose for PB in propylene glycol was 61.6 mg/kg (Tables 1 and 2). This was slightly lower than the literature value of 80 mg/kg (Pool and Hane, 1972; Latven and Sloane, 1954). The slope of the regression line for PB was 7.829 (± 2.119 se) and the Y-intercept was -8.997 (± 3.882 se).

The median lethal dose for permethrin in propylene glycol was determined to be 1000 mg/kg (Tables 1 and 2). The slope associated with the probit analysis was 1.803 (± 0.683 se) and the Y-intercept was -0.410 (± 2.001 se).

The lethal dose-response information obtained for DEET in this study was similar to that found in the literature. The median lethal dose following exposure to DEET was

3664 mg/kg (Table 1.2) while that reported in the literature was >3000mg/kg (Macko and Weeks, 1980). The slope associated with the probit analysis was 3.631 ($\pm 1.377se$) and the Y-intercept was -7.942 ($\pm 4.838se$).

Animals treated with propylene glycol alone appeared to be unaffected by this compound.

b. *Phase II*: Data obtained from the positive control group were not significantly different from phase I dose-response information. This indicates that the dosage levels selected were appropriate for the interaction portion of the study.

Table 1: Mortality associated with phase I. Dosage levels are spaced at 0.1 log intervals for PB and DEET and 0.2 log intervals for permethrin (Perm.).

Dosage (mg/kg)	Animals Treated	Pyidostigmine Mortality	Permethrin Mortality	DEET Mortality
PB 50	10	4	0	1
Perm. 316				
DEET 2000				
PB 63	10	3	5	3
Perm. 511				
DEET 2510				
PB 79	10	8	5	6
Perm. 794				
DEET 3160				
PB 100	10	10	6	4
Perm. 1260				
DEET 3980				
PB 126	10	10	6	7
Perm. 2000				
DEET 5010				

Table 2: Dosage levels used in phase II based on probit analysis of data from phase I.

Probit (% lethality)	PB mg/kg	Permethrin mg/kg	DEET mg/kg
4 (16%)	45.76	279	1946
4.5 (30%)	52.59	511	2628
5 (50%)	61.36	1000	3664
5.5 (70%)	71.59	1953	5109
6 (84%)	83.28	3576	6896

Table 3: Phase II study design: Expected mortality of dosage solutions when the vehicle contains the LD₁₆ (additive LD₃₂) of two compounds.

Dose	First Cmpd. LD	Second Cmpd. LD	Third Cmpd. LD	Additive Cmpds. LD
1	16%	16%	0%	32%
2	16%	16%	16%	48%
3	16%	16%	30%	62%
4	16%	16%	50%	82%
5	16%	16%	70%	>100%
6	16%	16%	84%	>100%

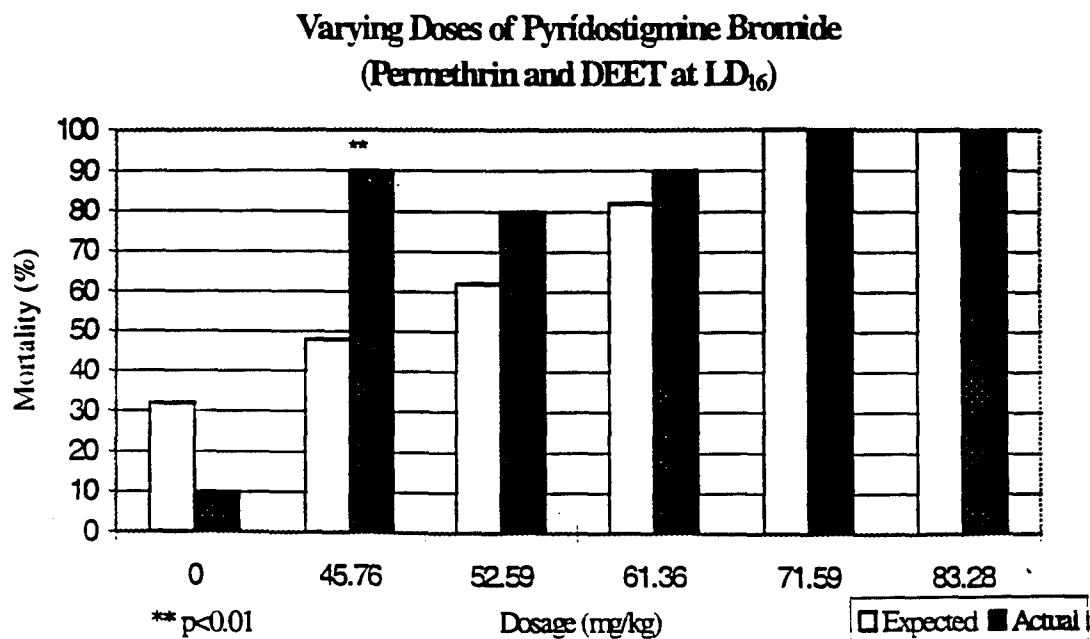


Figure 1: Response of rats given various doses of PB with LD₁₆ of permethrin and DEET (additive LD₃₂) as compared to expected additive mortality. (n=10 per dose).

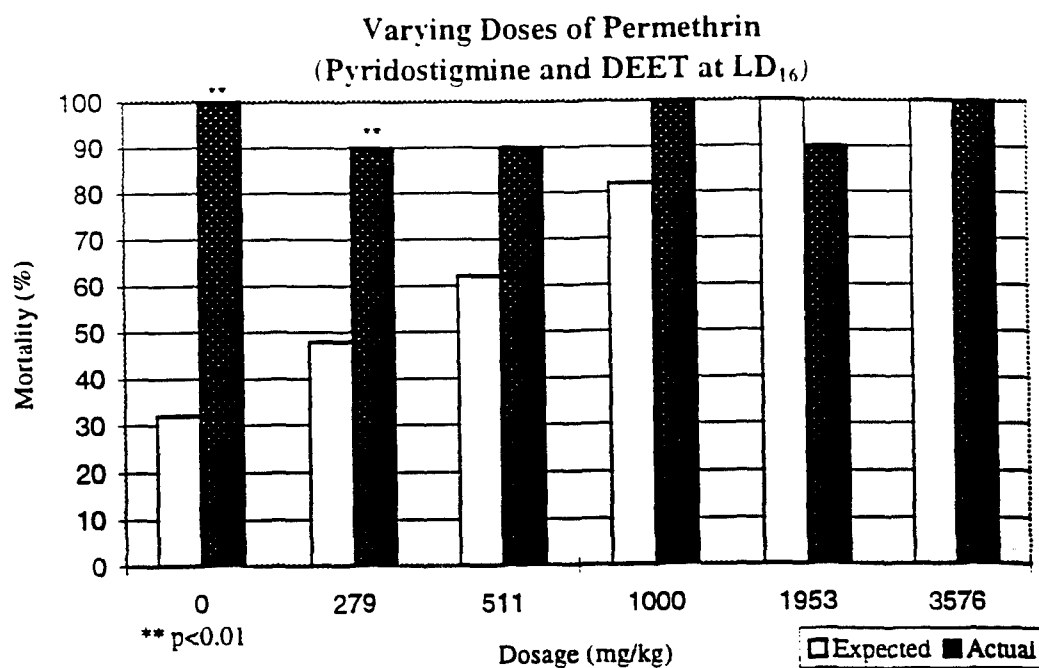


Figure 2: Response of rats given various doses of permethrin with LD₁₆ of PB and DEET (additive LD₃₂) as compared to expected additive mortality. (n=10 per dose).

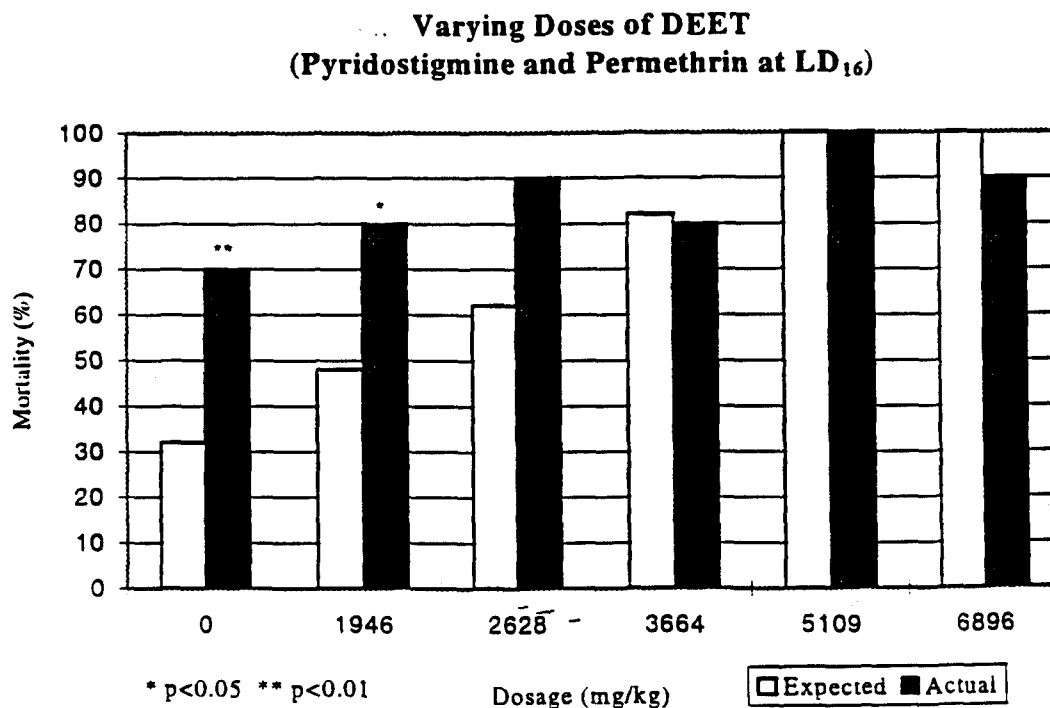


Figure 3: Response of rats given varying doses of DEET with LD₁₆ of PB and permethrin (additive LD₃₂) as compared to expected mortality. (n=10 per dose).

Summary of First Dosage Level

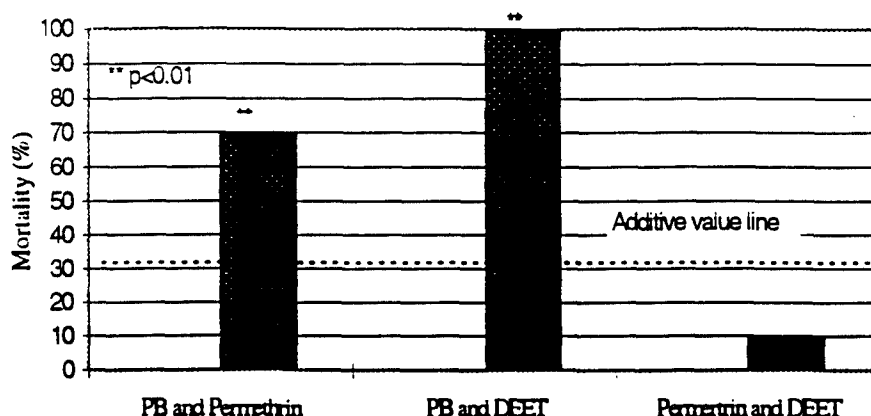


Figure 4: A comparison mortality associated with the first dosage level. Solutions contain LD₁₆ of two compounds (additive LD₃₂) only. The additive value line has been drawn in for contrast. (n=10 per group).

Solutions containing all three compounds produced higher than expected mortality where the additive value was less than 100% (Expected additive mortality values of dosage solutions are displayed in Table 3). The differences were significant where low mortality was expected (Figures 1, 2, and 3).

A comparison of dosage solutions containing LD₁₆ (additive LD₃₂) of only two compounds was also performed. The concentration of compounds in these vehicle solutions were expected to produce a 32% mortality. Solutions containing PB resulted in a significantly higher number of deaths than expected. The combination of PB and DEET resulted in the death of all exposed animals. The combination of PB and Permethrin also produced a significantly higher than expected number of deaths. The combination of permethrin and DEET resulted in a lower than expected number of deaths, however, this was not significant. The deaths associated with this solution was significantly lower than solutions containing PB (Figure 4).

7. DISCUSSION:

The results of this study indicate that a significant increase in lethality occurs when PB, permethrin, and DEET are given concurrently to male Sprague-Dawley rats by gavage. Furthermore, solutions containing PB and permethrin or PB and DEET also caused a significant increase in lethality when compared to expected additive values. This information suggests that lethality in this study was more than an additive effect.

This study provided new information on the lethal effect of permethrin when used in conjunction with the vehicle, propylene glycol. The literature value for the median lethal dose of technical grade permethrin is 4000 mg/kg, however, the value for permethrin in corn oil is 380 mg/kg (Metker *et al.* 1977). Metker's study also indicated

that the oral lethal dose for permethrin is highly correlated with age, nutritional state, gender, strain of rat used, and vehicle.

There are at least two possible mechanisms which, by concurrent oral exposure to compounds used in this study, could increase lethality. For instance, PB, which has a steep dose-response curve, is poorly absorbed by the gut. It is possible, therefore, that an increase in the bioavailability of PB could cause the increased lethality seen in this study. DEET, which has been used as a transdermal carrier molecule for the delivery of drugs and other agents (Hussain and Ritschel, 1988, Reifenrath *et al.* 1984; Windheuser *et al.*, 1982), may enhance the uptake of PB from the gut. This would increase the levels of PB in circulation and decrease the activity of esterases. Another possible mechanism is the inhibition of detoxification systems. For instance, esterase inhibition by PB could also inhibit the degradation of permethrin, an ester. Hydrolysis of the ester bond in permethrin is mediated by non-specific esterases (Ecobichon, 1991; Cassida *et al.*, 1983). Inhibition of this class of enzymes would effectively increase the residence time of permethrin in the body and may explain the increased lethality when these compounds are given simultaneously. Carbamates and pyrethroids are also degraded by cytochrome P-450 in the liver. This detoxification system may become overloaded with an increase in circulating levels of these toxicants. This would decrease the effectiveness of this enzyme system.

Further studies are necessary in order to understand the relationship among these compounds. Biopharmaceutic and pharmacokinetic studies would identify increases in blood levels of PB and decreases in esterase activity as well as alterations in clearance rates for compounds and metabolites. Neuropharmacological, neuropathological and neurobehavioral assessment is also necessary in order to determine if nonlethal endpoints are neurological in nature. Another possible study would examine various routes of administration associated with the use of these compounds. The most likely exposure to permethrin and DEET would occur through the dermal route while exposure to PB would occur orally.

It must be remembered that the information obtained through these studies is preliminary. This study used only one route of exposure in order to produce a quantifiable effect. The most likely human exposure scenario would be dermal exposure to permethrin and DEET and oral exposure to pyridostigmine. Mechanisms which caused increased lethality in this study may be partitioned if different routes of administration are used. Furthermore, dosage levels of compounds used in this study were sufficient to produce lethality following a single dose. For example, in order for an average 70kg (155lb) service member to become exposed to the lowest doses used in this study (PB = 46mg/kg, Permethrin = 279mg/kg, DEET = 1946mg/kg), this person would have to simultaneously ingest 107 PB tablets (30mg each), 23 six ounce cans of 0.5% permethrin aerosol spray, and 6.6 two ounce tubes of 33% DEET. Human exposure would most likely occur at low levels over an extended period of time. Another factor to consider is the assumption of concurrent use. For instance, less than 5% of the deployed units had distributional access to permethrin for uniform impregnation. Furthermore, entomologists assigned in the Gulf during the conflict indicated a very low usage of personal repellents, including DEET, even at times and in areas where mosquitos were present and biting. Also, the cool seasonal climatic

conditions which prevailed at the time of the war (January and February, 1991) resulted in the near absence of biting insects. In fact, insect biting rates dropped to insignificant or near zero levels in October and did not rise again until the following March. PB was taken for about two weeks at the start of the air war in mid January and again for a briefer period at the start of the ground war in mid February, a time when insect biting rates were extremely low. These factors indicate that the concurrent use of PB, permethrin, and DEET by service personnel was probably very low.

APPENDIX A REFERENCES

- Cassida, J.E., D.W. Gammon, A.H. Glockman, and L.J. Lawrence. Mechanism of selective action of pyrethroid insecticides. *Annu. Rev. Pharmacol. Toxicol.* 23:413-438. 1983.
- Clark, M.J. and F. Matsumura. Two different types of inhibitory effects of pyrethroids on nerve Ca^{++} and $\text{Ca}^{++}/\text{Mg}^{++}$ ATPase activity in the squid *Loligo pealei*. *Pesticide Biochem. Physiol.* 18:180-190. 1982.
- Cohan, S.L., J.L.W. Pohlmann, J. Mikszewski, and D.S.O'Doherty. The pharmacokinetics of pyridostigmine. *Neurology* 26:536-539. 1976.
- Daniel, W.W. Biostatistics: A Foundation for Analysis in the Health Sciences. John Wiley and Sons, New York. 1991.
- Ecobichon, D.J. Toxic effects of Pesticides. in Toxicology: The Basic Science of Poisons. M.O. Amdur, J. Doull, and C.D. Klaassen, eds. Pergamon Press, New York. 1991.
- Finney, D.J. Probit Analysis. third edition. (pp 145-146) Cambridge University Press, New York. 1979.
- Hane D. Acute toxicity studies in mice, rats, rabbits, and dogs with pyridostigmine bromide (Ro 1-5130) and Ro 1-5237/000. Internal Report. Roche. 1977.
- Hussain, A.S., and W.A. Ritschel. Influence of dimethylacetamide, N,N-diethyl-m-toluamide, and 1-dodecylazacycloheptan-2-one on *ex vivo* permeation of phosphonoformic acid through rat skin. *Methods Fund. Exp. Clin. Pharmacol.* 10(11):691-694. 1988.
- Latven, A. and A. Sloane. A toxicologic appraisal of mestinon bromide. Pharmacology Research, Inc. Internal report. 1954.
- Lim, P, A. Benitez, and R. Petesch. Assay of pyridostigmine bromide WR-250710BD, BM03894. Report No. 696. SRI International Project NO. 8504. 15 May 1990.
- Metker, L.W., R.A. Angerhofer, C.R. Pope, and K.C. Swentzel. Toxicological evaluation of 3-(phenoxyphenyl) methyl (+)-*cis, trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (permethrin). Study No. 51-0831-78. U.S. Environmental Hygiene Agency. 1977

Macko, J.A. and M.H. Weeks. Phase 6: Acute toxicity evaluation of N,N-diethyl-*meta*-toluamide (M-Det.). Study No. 71-51-0034-81. March. 1977 - January 1980. U.S. Army Environmental Hygiene Agency. 1980.

Pool, W. and D. Hane. Acute toxicity of pyridostigmine bromide (mestinon) in adult and neonatal rats. Roche Laboratories. Internal report. 1972.

Reifenrath, W.G., E.M. Chellquist, E.A. Shipwash, W.W. Jederberg, and G.G. Krueger. Percutaneous penetration in the hairless dog, weanling pig, and grafted athymic nude mouse: evaluation of models for predicting skin penetration in man. Br. J. Dermatol. 111(Suppl. 27):123-135. 1984.

Shih, M.L. and R.I. Ellin. Mechanism of Solid phase extraction of nanogram levels of pyridostigmine from plasma. Proc. Second Annual Symposium "Sample Preparation and Isolation Using Bonded Silicas" pp33-36. 1993.

Somani, S.M., J.B. Roberts, and A. Wilson. Pyridostigmine metabolism in man. Clinical Pharmacol Therap. 13(3):393-399. 1972.

Snodgrass, H.L. Migration of ^{14}C permethrin from impregnated military fabric. Study No. 75-51-0351-82. U.S. Environmental Hygiene Agency. 1982.

Taylor, P. Anticholinesterase agents. in Goodman and Gilman's The Pharmacological Basis of Therapeutics. eighth ed. A.G. Gilman, T.W. Rall, A.S. Niles, and P. Taylor, eds. Pergamon Press, New York. 1991.

U.S. Environmental Protection Agency. N,N-diethyl-*m*-toluamide (DEET): Pesticide Registration Standard. Office of Pesticide and Toxic Substances. Washington, D.C. 1980.

Verschoye, R.D., A.W. Brown, C. Nolan, D.E. Ray, and T. Lester. A comparison of the acute toxicity, neuropathology, and electrophysiology of N,N-diethyl-*m*-toluamide and N,N-dimethyl-2,2-diphenylacetamide in rats. Fundam. Appl. Toxicol. 18(1):79-88. 1992

Windheuser, J.J., J.L. Haslam, L. Caldwell, and R.D. Shaffer. The use of N,N-diethyl-*m*-toluamide to enhance the dermal and transdermal delivery of drugs. J. Pharmacol. Sci. 71(11):1211-1213. 1982.

World Health Organization. Environmental Health Criteria 94: Permethrin. Geneva. 1990.